City of Austin
Founded by Congress, Republic of Texas, 1839
Watershed Protection Department
P.O. Box 1088. Austin. Texas 78767

December 14, 2018

Ms. Paige Najvar
U.S. Fish and Wildlife Service
Austin Ecological Services Field Office
10711 Burnet Road, Suite 200
Austin, TX 78758
Ph: (512) 490-0057; ext. 229
Paige_Najvar@fws.gov

Dear Ms. Najvar:

Enclosed is the City of Austin's 2018 annual report for the U.S. Fish and Wildlife Service l0(a)1(A) permit (TE-833851) covering scientific activities affecting *Eurycea sosorum, E. waterlooensis*, and *E. tonkawae*.

Please do not hesitate to contact me should you require additional information at 512-974-2652, or Tom Devitt, Watershed Protection Department Environmental Scientist, at 512-974-6340.

Sincerely,

Mike Personett, Assistant Director
Watershed Protection Department

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2018 Annual Report
U.S. Fish and Wildlife Service Scientific Permit (TE-833851)

Reporting period: 2018
This report documents activities involving Barton Springs and Austin Blind salamanders (*Eurycea sosorum* and *E. waterlooensis*, respectively) by the City of Austin that are authorized under the above permit for 2018. Tables and figures are numbered by section.

TE-833851, Section S., Permit Condition 6: General Annual Reporting Requirements for Barton Springs and Austin Blind salamanders

1) Precise locations of previously undocumented surveyed areas
None.

2) Dates of surveys conducted
Please see # 4, below.

3) Survey methods
Barton Springs and Austin Blind salamander counts were conducted quarterly throughout the year at Parthenia, Eliza, Old Mill (Sunken Gardens) and Upper Barton springs. For each survey, the date, weather, type of flow (base flow or storm flow) and aquifer discharge are recorded by the U.S. Geological Survey station at Parthenia Spring. Each site was searched using a drive survey method where all non-embedded substrate is searched, except for at Old Mill Spring, where a timed survey is used due to the low abundance of salamanders at that site. Every individual salamander found was identified to species and categorized by total length (0–1", 1–2", >2") or measured from photographs. Photographic capture-recapture surveys were performed at all sites except Parthenia Spring. Salamanders are captured using small handheld dip nets, photographed, and released as soon as possible, usually within 1–4 hours. The total number of salamanders of each species and size class found were recorded, although we only present the totals below.

4) Survey results
Salamander counts from 2018 surveys are presented in Table 1, below.

Table 1. Barton Springs and Austin Blind salamander counts from 2018.

Date	Site	Number *E. sosorum*	Number *E. waterlooensis*
2/9/2018	Old Mill Spring	8	0
2/9/2018	Upper Barton Spring	5	0
2/12/2018	Eliza Spring	418	5
2/14/2018	Eliza Spring	498	8
2/16/2018	Eliza Spring	452	10
2/26/2018	Barton Springs	98	0
5/8/2018	Eliza Spring	365	1
5/11/2018	Eliza Spring	308	3
5/14/2018	Eliza Spring	313	4
5/17/2018	Barton Springs	211	0
5/23/2018	Old Mill Spring	9	0
5/23/2018	Upper Barton Spring	5	0
8/9/2018	Old Mill Spring	5	0
8/14/2018	Eliza Spring	427	0

8/17/2018	Eliza Spring	336	0
8/20/2018	Eliza Spring	312	0
8/21/2018	Upper Barton Spring	0 (no flow)	0 (no flow)
8/23/2018	Barton Springs Pool	165	0
10/30/2018	Eliza Spring	43	0
11/2/2018	Eliza Spring	38	0
11/5/2018	Eliza Spring	42	1
11/7/2018	Old Mill Spring	3	0
11/7/2018	Upper Barton Spring	6	0
11/15/2018	Barton Springs	9	0

5) <u>Number of salamanders collected from the wild</u>
Salamanders collected from the wild (salvaged from surveys or collected alive for genetic research) are presented in Table 2, below.

Table 2. Salamanders collected from the wild (N=41 *E. sosorum*, N=2 *E. waterlooensis*). Salvaged individuals were killed or injured during surveys, or otherwise found dead. Individuals that were collected alive were done so to serve as voucher specimens. All collected individuals have been deposited in the Biodiversity Collections at the University of Texas at Austin (TNHC) except for hatchlings and small juveniles that are being used to examine gut contents (marked NA).

Museum No.	Species	County	Locality	Date	Notes
NA	*sosorum*	Travis	Eliza Spring	2/12/18	Injured during survey; salvaged for gut contents
NA	*sosorum*	Travis	Eliza Spring	2/12/18	Injured during survey; salvaged for gut contents
NA	*sosorum*	Travis	Eliza Spring	2/12/18	Injured during survey; salvaged for gut contents
NA	sosorum	Travis	Eliza Spring	2/12/18	Injured during survey; salvaged for gut contents
NA	*sosorum*	Travis	Eliza Spring	2/12/18	Injured during survey; salvaged for gut contents
NA	*sosorum*	Travis	Eliza Spring	2/12/18	<1" TL, collected with injuries, recovering in captivity
NA	*sosorum*	Travis	Eliza Spring	2/14/18	Injured during survey; salvaged for gut contents
NA	*sosorum*	Travis	Eliza Spring	2/14/18	Injured during survey; salvaged for gut contents
NA	*sosorum*	Travis	Eliza Spring	2/14/18	Injured during survey; salvaged for gut contents
NA	*waterlooensis*	Travis	Eliza Spring	2/14/18	Injured during survey; salvaged for gut contents
NA	*sosorum*	Travis	Eliza Spring	2/14/18	<1" TL, collected with injuries
NA	*sosorum*	Travis	Eliza Spring	2/16/18	Injured during survey; salvaged for gut contents
NA	*sosorum*	Travis	Eliza Spring	2/16/18	Injured during survey; salvaged for gut contents
NA	*sosorum*	Travis	Eliza Spring	2/16/18	Injured during survey; salvaged for gut contents
NA	*sosorum*	Travis	Eliza Spring	2/16/18	Injured during survey; salvaged for gut contents
NA	*sosorum*	Travis	Eliza Spring	2/16/18	Injured during survey; salvaged for gut contents
NA	*sosorum*	Travis	Eliza Spring	2/16/18	Injured during survey; salvaged for gut contents
NA	*sosorum*	Travis	Parthenia Spring	2/26/18	1-2" total length, found dead during drawdown
TNHC 108522	*tonkawae*	Travis	SAS Canyon	3/1/18	collected for voucher/genetics
NA	*sosorum*	Travis	Eliza Spring	5/8/18	Injured during survey; salvaged for gut contents
NA	*sosorum*	Travis	Eliza Spring	5/8/18	Injured during survey; salvaged for gut contents
NA	*sosorum*	Travis	Eliza Spring	5/8/18	Injured during survey; salvaged for gut contents
NA	*sosorum*	Travis	Eliza Spring	5/8/18	Injured during survey; salvaged for gut contents
NA	*sosorum*	Travis	Eliza Spring	5/14/18	Injured during survey; salvaged for gut contents
NA	*sosorum*	Travis	Eliza Spring	5/14/18	Injured during survey; salvaged for gut contents
NA	*sosorum*	Travis	Eliza Spring	5/14/18	Injured during survey; salvaged for gut contents
NA	*sosorum*	Travis	Eliza Spring	5/14/18	Injured during survey; salvaged for gut contents
NA	*waterlooensis*	Travis	Eliza Spring	5/14/18	Injured during survey; salvaged for gut contents
NA	*sosorum*	Travis	Eliza Spring	5/14/18	Injured during survey; salvaged for gut contents
NA	*sosorum*	Travis	Eliza Spring	5/14/18	Injured during survey; salvaged for gut contents
NA	*sosorum*	Travis	Eliza Spring	5/14/18	Injured during survey; salvaged for gut contents
NA	*sosorum*	Travis	Eliza Spring	5/14/18	Injured during survey; salvaged for gut contents
NA	*sosorum*	Travis	Eliza Spring	5/14/18	Injured during survey; salvaged for gut contents
NA	*sosorum*	Travis	Old Mill Spring	5/23/18	accidental collection in invertebrate collection
NA	*sosorum*	Travis	Eliza Spring	8/14/18	Injured during survey; salvaged for gut contents
NA	*sosorum*	Travis	Eliza Spring	8/14/18	Injured during survey; salvaged for gut contents
NA	*sosorum*	Travis	Eliza Spring	8/17/18	Injured during survey; salvaged for gut contents
NA	*sosorum*	Travis	Eliza Spring	8/17/18	Injured during survey; salvaged for gut contents
NA	*sosorum*	Travis	Eliza Spring	8/17/18	Injured during survey; salvaged for gut contents

NA	*sosorum*	Travis	Eliza Spring	8/17/18	Injured during survey; salvaged for gut contents
NA	*sosorum*	Travis	Eliza Spring	8/20/18	Injured during survey; salvaged for gut contents
NA	*sosorum*	Travis	Eliza Spring	11/5/18	Injured during survey; salvaged for gut contents

6) Results of species identifications
See Table 1, above.

7) Number of salamanders handled and marked with elastomers
None

8) Observations of abnormal behavior or condition of salamanders handled/marked
None

9) Results of any mark-recapture work
We conducted capture-recapture surveys at three sites in 2018 using photographic identification methods (Bendik et al. 2013). Not enough recaptures were made at Upper Barton or Old Mill springs, so we are unable to calculate estimates of abundance at those sites.

We conducted robust-design mark-recapture sampling at Eliza Spring in February, May, August, and November. Abundance estimates are provided in Table 4, below.

Table 4. Estimates of abundance ($\widehat{N}$) and standard deviation (SD) for four capture-recapture surveys at Eliza Spring in 2018.

Period	$\widehat{N}$	SD
Feb-18	959.9509	26.9712407
May-18	874.1804	34.0923608
Aug-18	765.745625	22.972104
Nov-18	204.969725	32.7269703

10) Results of genetic research conducted as a result of tail-clipping
Six tail-tips were collected in 2018 (Table 5). Preliminary results of genetic analyses for *E. sosorum* and *E. waterlooensis* are provided in Appendix 1.

Table 5. List of tail-tip samples collected for genetic work. TJD=Thomas James Devitt.

Field No.	Species	County	Locality	Date	Notes
TJD 1197	*sosorum*	Travis	Upper Barton Spring	2/9/18	tail-tipped for genetics and released
TJD 1198	*sosorum*	Travis	Upper Barton Spring	2/9/18	tail-tipped for genetics and released
TJD 1199	*sosorum*	Travis	Upper Barton Spring	2/9/18	tail-tipped for genetics and released
TJD 1200	*sosorum x waterlooensis*	Travis	Eliza Spring	2/16/18	tail-tipped for genetics and released
TJD 1205	*sosorum*	Travis	Backdoor Spring	3/21/18	tail-tipped for genetics and released
TJD 1206	*sosorum x waterlooensis*	Travis	Eliza Spring	3/9/18	tail-tipped for genetics and released

11) Results of any research or management activities authorized by this permit and approved through the submission of study plans to the CPI Branch of the Austin ESFO

a. City of Austin monitors water quality in the Barton Springs Complex under this permit to meet the requirements of the Habitat Conservation Plan contained in the USFWS l0(a)(l)(B) permit PRT-839031and the Texas Pollutant Discharge Elimination System permit WQ0004705000 (EPA NPDES TXS000401). Permitted staff collect water samples from each spring in the Barton Springs complex. On an approximately biweekly frequency, tested parameters include total suspended solids, volatile suspended solids, $N03+N02$-N, $NH3$-N,

Ortho-P, temperature, dissolved oxygen, pH, conductivity, and turbidity. Quarterly sampling includes biweekly parameters plus alkalinity, Ca, Na, K, Mg, Cl, S04, F, As, Cu, Fe, Pb, Ni, Zn. TPDES annual sampling includes all of the above plus Hardness, Ag, Cd, Cr, Hg, TOC, oil and grease, total polycyclic aromatic hydrocarbons, bromacil, organophosphate pesticides, chlorinated herbicides, volatiles, and semi-volatiles. Additionally, the City of Austin in cooperation with the United States Geological Survey maintains continuous monitoring for spring discharge and physiochemical parameters at Barton Springs.

b. U.S. Geological Survey deploys and maintains water quality sampling equipment in Parthenia Spring. Equipment was serviced by USGS dive teams.

c. City of Austin staff collect sediment samples at the four Barton springs for testing to meet requirements of the City's TPDES permit. Samples were collected on 4/19/18 at all four spring sites (Eliza, Old Mill, Upper Barton, and Barton Springs Pool), and on 2/1/18, 7/24/18, and 12/6/18 at Barton Springs Pool only.

TE-833851 Permit Condition: Captive Breeding Annual Reporting Requirements

1) The number of *Eurycea sosorum*, *E. waterlooensis*, and *E. tonkawae* held at the captive breeding facility (including the number of wild-caught and captive-bred individuals from each spring site collected).

Table 1. Inventory of salamanders in the captive breeding program. WC=wild caught, CB=captive bred.

Species	Spring of Origin	WC	CB>6 mo.
Eurycea sosorum	Parthenia	6	41
	Old Mill	4	148
	Eliza	25	68
	UBS	0	5
	Dallas Aquarium[1]	0	1
Total		**35**	**263**
E. waterlooensis	Parthenia	0	NA[2]
	Old Mill	5	NA[2]
	Eliza	1	NA[2]
	UBS	0	NA[2]
Total		**6**	**39**
E. tonkawae	Bull Creek	3	4
	McDonald Well	0	5
	SAS Canyon	1	0
	Testudo Tube	2	0
	Wheless	2	0
Total		**8**	**9**

[1] Founder salamanders for the Dallas Aquarium captive population were collected from more than one spring site (Parthenia and Old Mill) and mixed together. COA has F2's from Dallas F1's that were used for educational purposes at the Splash! Into the Edwards Aquifer exhibit at Barton Springs in Zilker Park.
[2] *E. waterlooensis* are not separated and bred according to spring site of origin due to the fact that the species is primarily aquifer-dwelling.

2) Number of observations of courtship behavior, spermatophores, spermatophore depositions, sperm transfers, and ovipositions.

In 2018, courtship behavior was observed in both wild-caught and captive-bred salamanders. In general, salamanders are not disturbed by City staff during courtship. Because salamanders can store sperm, observed courtship behavior does not necessarily result in immediate egg-laying. Each oviposition with viable offspring represents at least one sperm transfer, and possibly multiple transfers. Oviposition data are presented in Table 2.

Table 2. Ovipositions in captivity 12/01/17-11/30/18. Tank ID indicates spring site of origin, reproductive group, and wild-caught or captive-bred status. Individuals in reproductive groups are recorded in order to follow actual or potential dams and sires. BSP denotes groups from Parthenia Spring, E, groups from Eliza Spring, OM, groups from Old Mill Spring, UBS, groups from Upper Barton Spring, and F, captive-bred salamanders.

Estimated Oviposition Date	Tank ID	Clutch Size	No. Hatched
	Eurycea sosorum		
01/23/18	OMF1 (C176)	34	NA[1]
01/30/18	BSPF1 (C285)	14	NA[1]
02/21/18	E (C304)	16	Did not develop
02/22/18	OMF1 (C231)	24	15
03/04/18	BSPF1 (C273)	22	4
03/12/18	OMF2 (C230)	21	Did not develop (not fertilized)
04/17/18	OMF1 (C231)	13	11

[1] Eggs preserved to manage the population size and genetic diversity (prevent a disproportionate number of offspring produced from a single reproductive group, or to minimize inbreeding)

3) Information on clutch sizes (range, mean, and standard deviation) and hatching success (range, mean, and standard deviation)

Table 3. Salamander clutch size and hatching success for *E. sosorum* from 12/01/17–11/30/18.

	Range	Mean	Standard Deviation
Clutch Size	13-34 (N=7)	20.6	7.25
No. Hatched	0-15 (N=4)	7.5	6.76
% Hatched	0-85 (N=4)	41.3	39.0

4) Salamander Mortalities (including age and cause of death, if known)

Table 4. Salamander mortalities from 12/01/17–11/30/18.

Species	Wild-Caught or Captive-Bred	Age (years)	No. Mortalities	Cause of Death (health condition observed)
E. sosorum	WC	10-13*[1]	6	Senescence
	WC	18	2	Senescence
	CB	3-5	4	Unknown
	CB	5-7	9	Unknown
	CB	7-9	22	Senescence
	CB	9-11	10	Senescence
	CB	11-13	3	Senescence
E. waterlooensis	WC	3.5*[1]	1	Unknown
	CB	1	1	Unknown
	CB	10.5	1	Senescence
E. tonkawae	WC	13-141	5	Senescence

[1] Age of wild-caught salamanders is estimated based on size at collection, with a maximum estimated age of 1.5 years for salamanders > 2 inches total length at collection.

5) Information on Obvious Health Conditions or Behavioral Aberrations

No novel health conditions or behavioral aberrations were observed.

6) Special Projects

The captive breeding program provides support and salamanders for the public display tank at the Splash! Into the Edwards Aquifer Educational Exhibit. In addition, with prior approval from U.S. Fish and Wildlife Austin Ecological Service, COA donated 6 female captive-raised *Eurycea sosorum* and 1 female captive-raised *E. waterlooensis* to the Museum of Living Art at the Fort Worth Zoo for display purposes.

TE-833851, Section T, Permit Condition 6: General Annual Reporting Requirements for Jollyville Plateau Salamanders

In collaboration with Travis County Transportation and Natural Resources, we surveyed 76 sites along Long Hollow Creek and three tributaries of Cypress Creek, Travis County, in 2018 for presence of Jollyville Plateau salamanders. Each site was visited three times (unless the site was dry on the first visit) between 2/28/2018 and 3/19/2018. 34 of sites were dry, while 42 were wet. We only observed salamanders at the following five sites, all of which were in the Cypress Creek watershed:

SAMPLE_ID	LATITUDE	LONGITUDE
Site 159	30.42196	-97.8499
Site 161	30.44997	-97.8539
Site 145	30.43546	-97.857
Site 195	30.45569	-97.8411
Site 144	30.43516	-97.8584

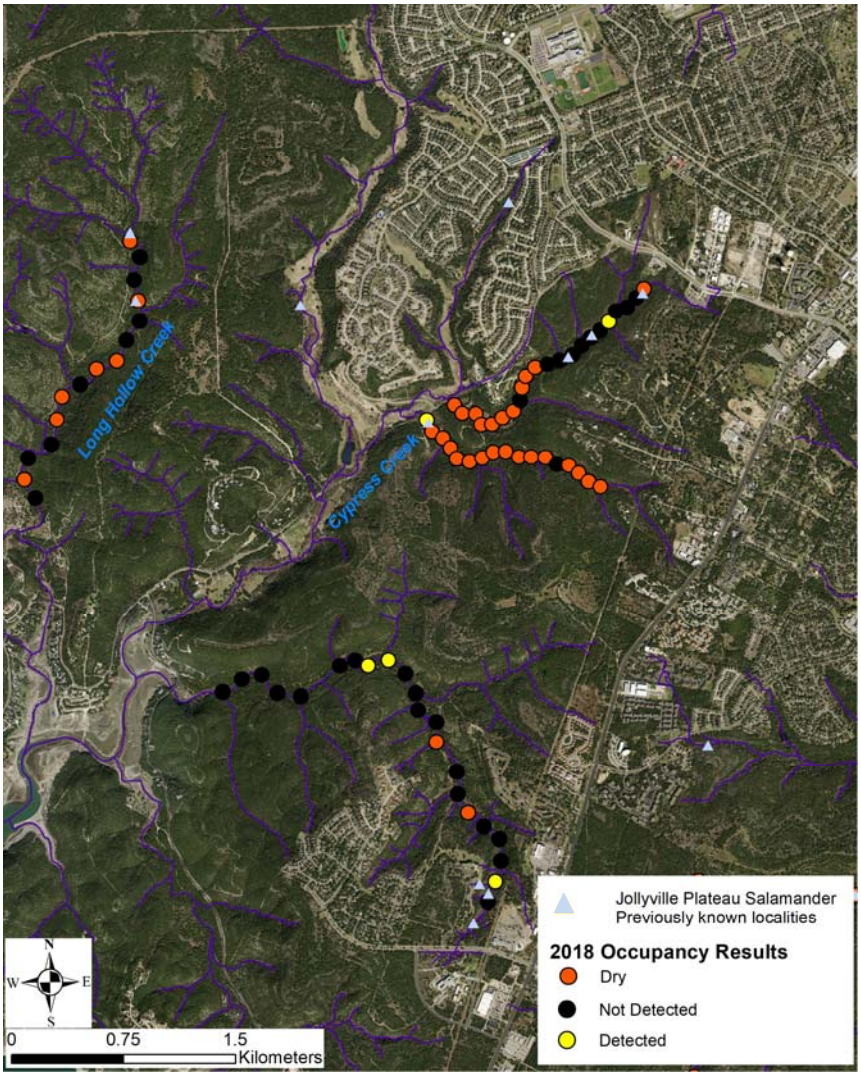

Figure 1. Map of the Cypress Creek and Long Hollow Creek watersheds showing the location of occupancy surveys for *E. tonkawae* in 2018.

TE-833851, Section U, Permit Condition 6: General Annual Reporting Requirements for San Marcos Salamanders

No research activities were performed on San Marcos salamanders (*Eurycea nana*) or within San Marcos Salamander habitat.

TE-833851, Section V, Permit Condition 6: General Annual Reporting Requirements for Karst Invertebrates

During the course of hydrogeological work, City of Austin permitted staff entered several caves that may harbor protected karst invertebrates. These caves, the dates of entry, City personnel, purpose of visit, and relevant observations are presented in the table below.

Date	Cave or Property	Purpose	Habitat Observations	Karst Zone	Personnel
1/18/2018	SAS Institute, 11920 Wilson Parke Ave. Development project.	Karst survey for recharge features	1 known cave entrance: SAS salamander cave	1	Sylvia Pope, Yazmin Avila, Saj Zappitello, Lindsey Sydow
3/19/2018	Grassy Cove Cave	Air quality monitoring prior to educational trip	Cave environment and habitat conditions as usual. Low potential for endangered species due to karst zone 3.	3	Lindsey Sydow, Saj Zappitello, Jessica Gordon
3/20/2018	SAS Institute, 11920 Wilson Parke Ave. Development project.	Karst survey for recharge features	No cave entrances found, no habitat encountered	1	Sylvia Pope, Yazmin Avila, Lindsey Sydow
4/10/2018	SAS Institute, 11920 Wilson Parke Ave. Development project.	Karst survey for recharge features	No cave entrances found, no habitat encountered	1	Sylvia Pope, Yazmin Avila, Lindsey Sydow
6/28/2019	LaCrosse Cave	Educational tours for families	Cave environment and habitat conditions as usual. Low potential for endangered species due to karst zone 3.	3	Lindsey Sydow
7/3/2018	SAS Institute, 11920 Wilson Parke Ave. Development project.	Karst survey for recharge features	No cave entrances found, no habitat encountered	1	Scott Hiers, Lindsey Sydow, Yazmin Avila, Saj Zappitello
7/3/2018	McNeil High School	Inspection of karst voids	Karst voids inspected, encountered during construction, potential habitat observed. Consultant to conduct environmental surveys.	1	Scott Hiers, Yazmin Avila, Saj Zappitello

Date	Cave or Property	Purpose	Habitat Observations	Karst Zone	Personnel
7/3/2018	Heritage Oaks/Pearson Ranch	Inspection of karst voids	Karst void/cave inspected, encountered during construction, potential habitat observed. Consultant to conduct environmental surveys.	1	Scott Hiers, Yazmin Avila, Saj Zappitello
7/6/2018	Tru by Hilton Arboretum. Development project. 11603 Jollyville Rd	Karst survey for recharge features	One potential cave entrance found - asked applicant to investigate further. Hand excavated to compacted clay and/or bedrock. No open conduit observed	1	Lindsey Sydow
8/10/2018	Mopac at La Crosse intersection	Inspection of karst voids/caves encountered during construction	Karst voids/caves encountered during construction. TxDOT to coordinate with consultant for environmental surveys.	3	Saj Zappitello
8/22/2018	9804 FM 620. Proposed acquisition.	Karst survey for recharge features	Some rock piles, did not lead to subsurface karst. No habitat encountered.	1	Scott Hiers, Lindsey Sydow, Saj Zappitello
9/18/2018	Covert Ford detention pond, Jollyville Road	Inspection of potential karst void	Small karst void at bottom of pond. Void to be protected for water quality. BCCP staff notified for species concerns. Could lead to potential karst invertebrate habitat, much too small to enter.	1	Saj Zappitello
9/20/2018	Divide Swamp Sinkhole	Recharge investigation	Assessment of recharge potential of sinkhole. Low potential for endangered species due to karst zone 3.	3	Scott Hiers, David Johns, Lindsey Sydow, Yazmin Avila, Saj Zappitello
9/24/2018	Doe Meadow Dr water quality pond.	Inspection of potential karst void	Small karst void intersected by old boreholes in bottom of pond. Void to be protected for water quality, low potential for endangered species due to karst zone 3.	3	Saj Zappitello
10/17/2018	Great Hills Baptist Church, Jollyville Road.	Inspection of potential karst void	Void was non-karst scour feature. No habitat encountered.	1	Saj Zappitello
11/16/2018	Mopac at La Crosse intersection	Inspection of karst voids/caves encountered during construction	Karst voids/caves encountered during construction. TxDOT to coordinate with consultant for environmental surveys.	3	Saj Zappitello

Date	Cave or Property	Purpose	Habitat Observations	Karst Zone	Personnel
11/30/2018	Bowie Cave	Education and geologic observation	Cave environment as usual. No species observed, low potential for endangered species due to Karst Zone 3	3	Lindsey Sydow, Saj Zappitello, Radmon Rice
12/10/2018	Stormwater Pond at Taylor Draper and Penny Creek	Inspect small feature where gabion filter has been sinking despite repairs	Small karst feature located under gabion filter wall in water quality pond. Difficult to excavate or measure full extent due to gabion wall. No open conduit or habitat observed.	2	Lindsey Sydow

Appendix 1. Conservation Genetics of the Barton Springs Salamander (*Eurycea sosorum*) Part I: Population Structure and Hybridization with *E. waterlooensis*

Tom Devitt

ABSTRACT

The Barton Springs Salamander Recovery Plan put forth by the U.S. Fish and Wildlife Service and the City of Austin's Habitat Conservation Plan both detail specific actions for ensuring the long-term persistence of this species. These actions include characterizing population genetic variation in the wild to evaluate the role of genetic factors in extinction risk and guide recovery efforts. Here, we present preliminary results from a fine-scale population genetic analysis of *Eurycea sosorum* including newly-discovered populations outside of Barton Springs to quantify population structure, as well as to investigate hybridization with *E. waterlooensis*. Population structure was well-defined, with *E. sosorum* individuals strongly assigned to eastern and western clusters. There was little genetic differentiation among the four Barton Springs, though Upper Barton Springs showed some allele frequency differences from Old Mill, Parthenia, and Eliza springs. Hybridization between *E. sosorum* and *E. waterlooensis* appears to be infrequent and restricted to Old Mill, Eliza, and Parthenia springs. However, hybrids are viable and fertile, given the presence of F1, F2, and both backcross classes that were observed in the sample. Future analyses include inferring ancestral population sizes and migration rates among subpopulations of *E. sosorum*.

INTRODUCTION

The Barton Springs Salamander Recovery Plan put forth by the United States Fish and Wildlife Service (U.S. Fish and Wildlife Service 2016) details specific actions for recovering (i.e., downlisting or delisting) this species. These actions include characterizing population genetic variation in the wild to evaluate the role of genetic factors (e.g., inbreeding depression, loss of adaptive variation through drift) in extinction risk and to guide recovery efforts. Although a recovery plan has not been drafted for *E. waterlooensis*, an interim conservation strategy for this species should mirror the recovery strategy set forth in the Barton Springs Salamander Recovery Plan (U.S. Fish and Wildlife Service 2016). We view this recovery plan as a guide for action and tool for measuring progress. Here, we begin to address two specific recovery actions for *E. sosorum*:

Action 4.1.3	Determine gene flow and migration between the four spring sites and genetic variation within and among sites
Action 4.1.6	Investigate the genetic characteristics and variation in the Barton Springs Salamander at the individual and population level

Sample size limitations for *E. waterlooensis* will preclude the same level of detailed inference that is possible with *E. sosorum*, though including available samples will still yield important new insights.

Results of this work will provide a better understanding of the population biology of the covered species to inform habitat management, captive breeding, and conservation.

METHODS

Sampling. We sampled 164 salamanders from 15 sites in the Barton Springs segment of the Edwards Aquifer (Fig. 1). For localities where voucher specimens exist, we collected a non-lethal tissue sample from the tip of the tail for genetic analysis. For newly-discovered populations, 2–3 individuals were collected and preserved following standard methods for salamanders (Jacobs and Heyer 1994; McDiarmid 1994) and deposited in the Biodiversity Collections (formerly Texas Natural History Collections; TNHC) at the University of Texas at Austin. Samples were stored in liquid nitrogen at the TNHC prior to DNA extraction and sequencing. Salamanders were captured by hand with aquarium nets, aquatic drift net traps placed over spring outlets, and artificial cover near springs (cotton mopheads) (Devitt and Nissen 2018; Holsinger and Minckley 1971; Gibson, Harden, and Fries 2008). Collections were made under scientific collecting permits from the United States Fish and Wildlife Service (TE833851-4) and Texas Parks and Wildlife Department (SPR-0113-006).

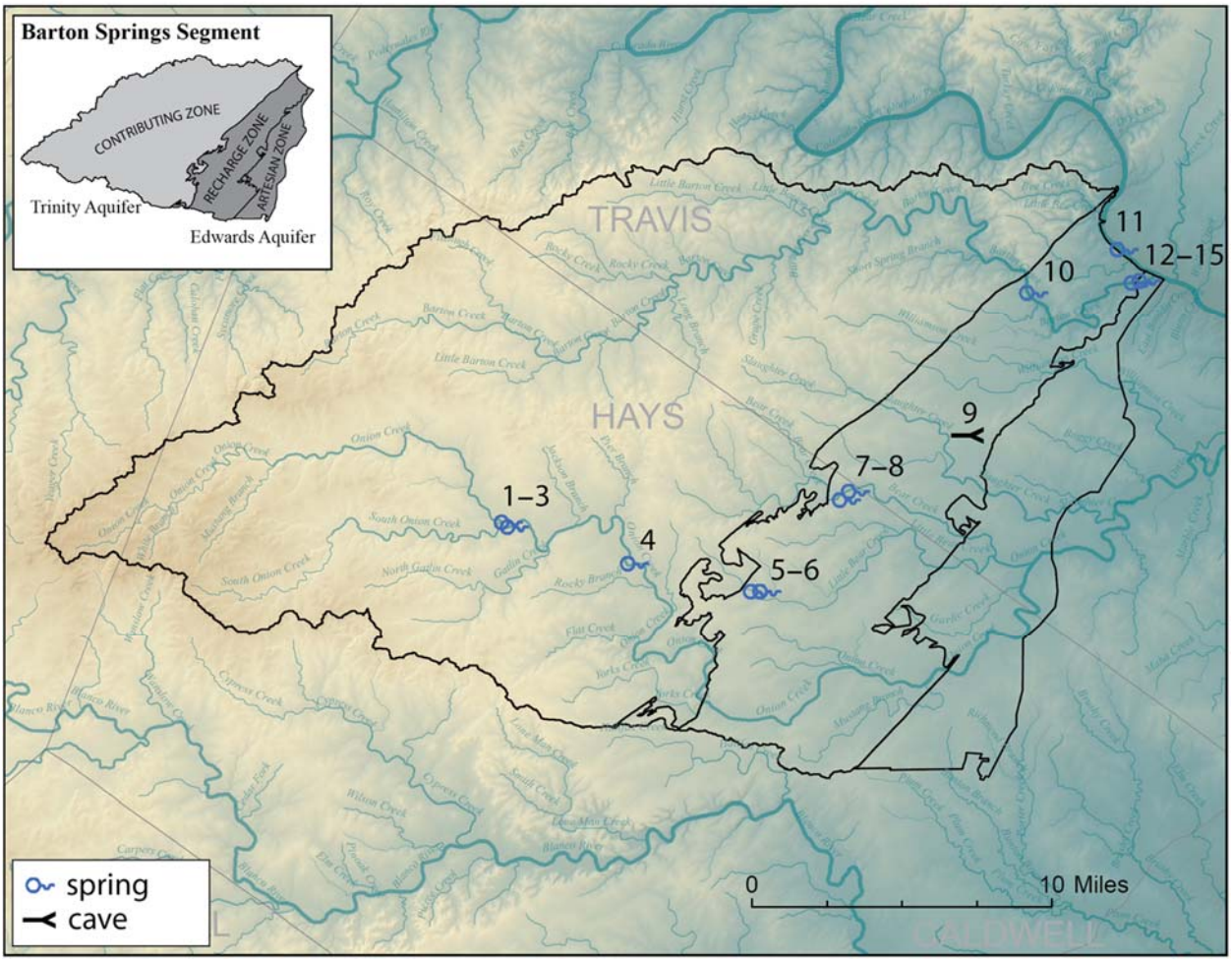

Figure 1. Sampling locations for Barton Springs salamanders collected for this study. 1) Emerald Spring; 2) Bello

Spring; 3) Pearly's Spring; 4) Ben McCulloch Spring; 5) Taylor Spring; 6) Stuart Spring; 7) Spillar Ranch Spring 1; 8) Spillar Ranch Spring 2; 9) Blowing Sink Cave; 10) Backdoor Spring; 11) Cold Spring; 12) Upper Barton Spring; 13) Parthenia Spring; 14) Eliza Spring; and 15) Old Mill Spring.

DNA Sequence Data Collection. To discover loci and genotype individuals, we used double-digest restriction-site associated DNA sequencing following the protocol of Peterson et al. (2012). Library preparation and sequencing was performed by the Genomic Sequencing and Analysis Facility at the University of Texas at Austin. Briefly, whole genomic DNA was first isolated from tissue samples using silica-based spin columns (Qiagen). DNA extracts were quantified using fluorometry (Qubit, ThermoFisher Scientific) and normalized to a concentration of 10 ng/ul. Restriction endonuclease digests of 100 ng of genomic DNA was performed using a 6-base common cutter (SphI) and an 8-base rare cutter (SbfI) in a single reaction following the manufacturer's protocol (New England BioLabs). Digests were purified using solid phase reversible immobilization (SPRI) beads following the manufacturer's protocol (Agencourt AMPure XP system, Beckman Coulter) and quantified using fluorometry. Twenty microliters of each digest was individually barcoded by ligating a combinatorial in-line adapter and a standard Illumina multiplexing read index onto DNA fragments. Oligonucleotide sequences for the adapters and corresponding PCR primers for the multiplexing read indices are provided in Peterson et al. (2012). Following ligation, samples were pooled by similar DNA concentration (up to 29 samples per pool) into 8 pools. Each pool of ligation products was then size-selected for 300-bp fragments (excluding the 76 bp of adapter added during ligation) using pulsed-field electrophoresis (BluePippin, Sage Science). Illumina sequencing libraries were generated using PCR amplification with high-fidelity DNA polymerase (Phusion, New England BioLabs). PCR amplicons were cleaned with SPRI beads and analyzed to quantify molarity and library fragment size distribution using an Agilent BioAnalyzer (Agilent Technologies). Finally, samples were sequenced on the Illumina HiSeq 2500 platform (100 bp paired-end run).

Data Assembly. We used the software pipeline *ipyrad* (https://github.com/dereneaton/ipyrad) to filter and sort reads, identify loci *de novo* and genotype individuals. Five samples were excluded from data assembly due to too few reads or low overall sequence quality. A range of values were used for parameters to optimize assemblies because these parameters may affect downstream analyses and resulting inference (Mastretta-Yanes et al. 2014; Ilut, Nydam, and Hare 2014; Harvey et al. 2015). Different subsets of individuals were assembled to investigate population structure and hybridization at different geographic scales. RAD data assembly was performed on the Lonestar 5 high performance computing system at the Texas Advanced Computing Center, University of Texas, Austin.

Population Structure and Hybrid Identification. We used the Bayesian clustering method implemented in *Structure* 2.3.4 (Pritchard, Stephens, and Donnelly 2000) to infer population structure, estimate population allele frequencies at each locus, and identify hybrid *E. sosorum* x *E. waterlooensis* individuals. We used the correlated allele frequencies model and assumed admixture among populations using default parameters for the hyperparameters λ, α and F and the priors used to parameterize the probability models, as recommended by the software authors. For each value of K, ten replicate runs consisting of 100,000 sweeps after a burn-in period of 10,000 sweeps were performed. We evaluated the highest level of population structure by examining the log probability of the data ($\ln Pr(X|K)$) as recommended by the software authors, as well as the delta K statistic of Evanno et al. (Evanno, Regnaut, and Goudet 2005). When clusters were found, we explored further population subdivision by performing additional analyses within clusters following the software authors' recommendation (section 5.3 in Pritchard, Wen, and

Falush 2010). We used the program *StrAuto* (Chhatre and Emerson 2017) to automate analyses on the Lonestar 5 HPC. Results were summarized and visualized using *Clumpp* (Jakobsson and Rosenberg 2007) and *Distruct* (Rosenberg 2003) as implemented in the package *pophelper* 1.2.1 (Francis 2017) for the R software environment (R Core Team 2018). Plots of ΔK and $\ln\Pr(X|K)$ were constructed in the web program Structure Harvester (Earl and vonHoldt 2012).

Hybrid Identification and Classification. For individuals identified in *Structure* as admixed, we used the software *NewHybrids* (Anderson and Thompson 2002) as implemented in the R packages *parallelnewhybrid* (Wringe et al. 2016) and *hybriddetective* (Wringe et al. 2017) to classify individuals as one of 6 distinct genotype frequency classes (2 parentals, F1s, F2s, 2 backcrosses) that result from early generation matings between distinct species. We first used the program PGDSpider (Lischer and Excoffier 2011) to convert the input file to the *NewHybrids* format. We used the default genotype categories for first- and second-generation crossings and ran 5 replicate runs of 100,000 sweeps after a burn-in of 50,000 sweeps using a Jeffrey's-like prior for the mixing proportions (π) and allele frequencies (θ).

PRELIMINARY RESULTS

Data Assembly. Population structure analyses were based on three primary assemblies (Table 1) comprising different subsets of individuals. Our largest assembly (sos_wat-min140) included 159 individual *E. sosorum* and *E. waterlooensis* sampled from throughout the species' ranges. Based on *Structure* analysis of this dataset (see below), a subset of those samples (90 individuals from the Barton Springs complex only; dataset sos_wat-min140_BS) were analyzed in *NewHybrids* to classify hybrids. Finally, a third assembly consisting of 139 "pure" parental *E. sosorum* individuals only (sosorum-min130) was analyzed separately.

Table 1. Data assemblies.

Dataset	Num. individuals	Min. samp/loc	Num. loci
sos_wat-min140	159	140	702
sos_wat-min140_BS	90	140*	702
sosorum-min130	139	130	588

*The minimum number of samples per locus parameter is greater than the number of individuals in this assembly because individuals were pruned from a more inclusive dataset (sos_wat-min140) containing the same loci.

Population Structure and Hybrid Classification. At the highest level of population structure, *Structure* analysis of the entire dataset revealed the presence of two distinct clusters (i.e., K=2) corresponding to *E. sosorum* and *E. waterlooensis* (Fig. 2A). Six individuals from the Barton Springs complex were identified as admixed (Fig. 2A). *NewHybrids* analysis of the three sites where *E. waterlooensis* individuals or hybrids were detected were identical across 5 separate runs, classifying 1 individual as an F1 hybrid, 2 as F2s, 1 as backcross to *E. sosorum*, and 2 as backcross to *E. waterlooensis* (Fig. 2B). Removal of *E. waterlooensis* and hybrid individuals revealed two main clusters of populations at the highest level of population structure, one in the southwestern portion of the range and one in the northeastern portion of the range including Barton Springs (Figs. 3 and 4). At levels of K>2, Onion Creek populations are recovered as a distinct cluster in about half of the runs (Figs. 3 and 4).

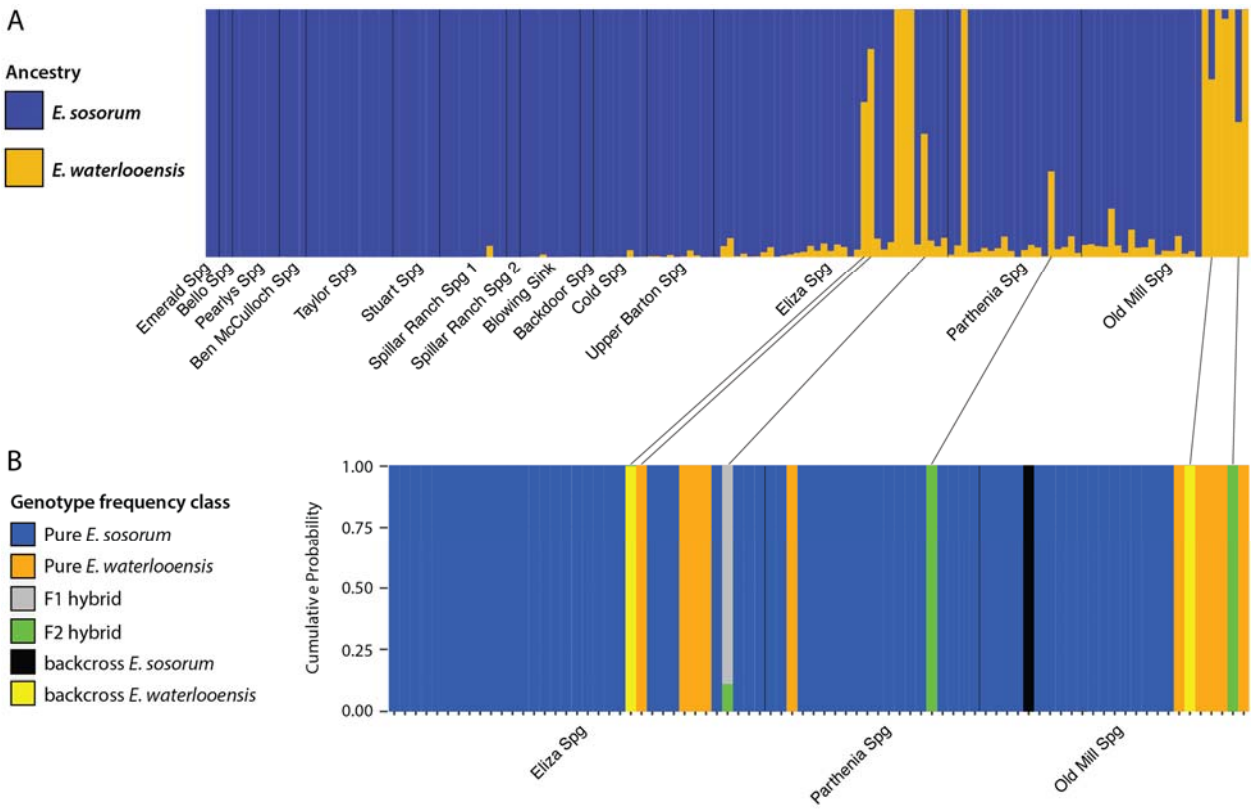

Figure 2. A) Summary plot of ancestry estimates from *Structure* analysis of 159 individual *E. sosorum* and *E. waterlooensis* genotyped at 702 loci. Each individual in the dataset is represented by a single vertical bar that is colored by that individual's estimated membership proportion in each of *K* inferred clusters (here, *K*=2). B) Summary plot of ancestry estimates from *NewHybrids* analysis of 80 individual *E. sosorum* and *E. waterlooensis* from Eliza, Parthenia, and Old Mill springs genotyped at 702 loci. Each vertical bar represents an individual, with the height of the color reflecting the cumulative probability of that sample falling into each of 6 distinct genotype frequency classes.

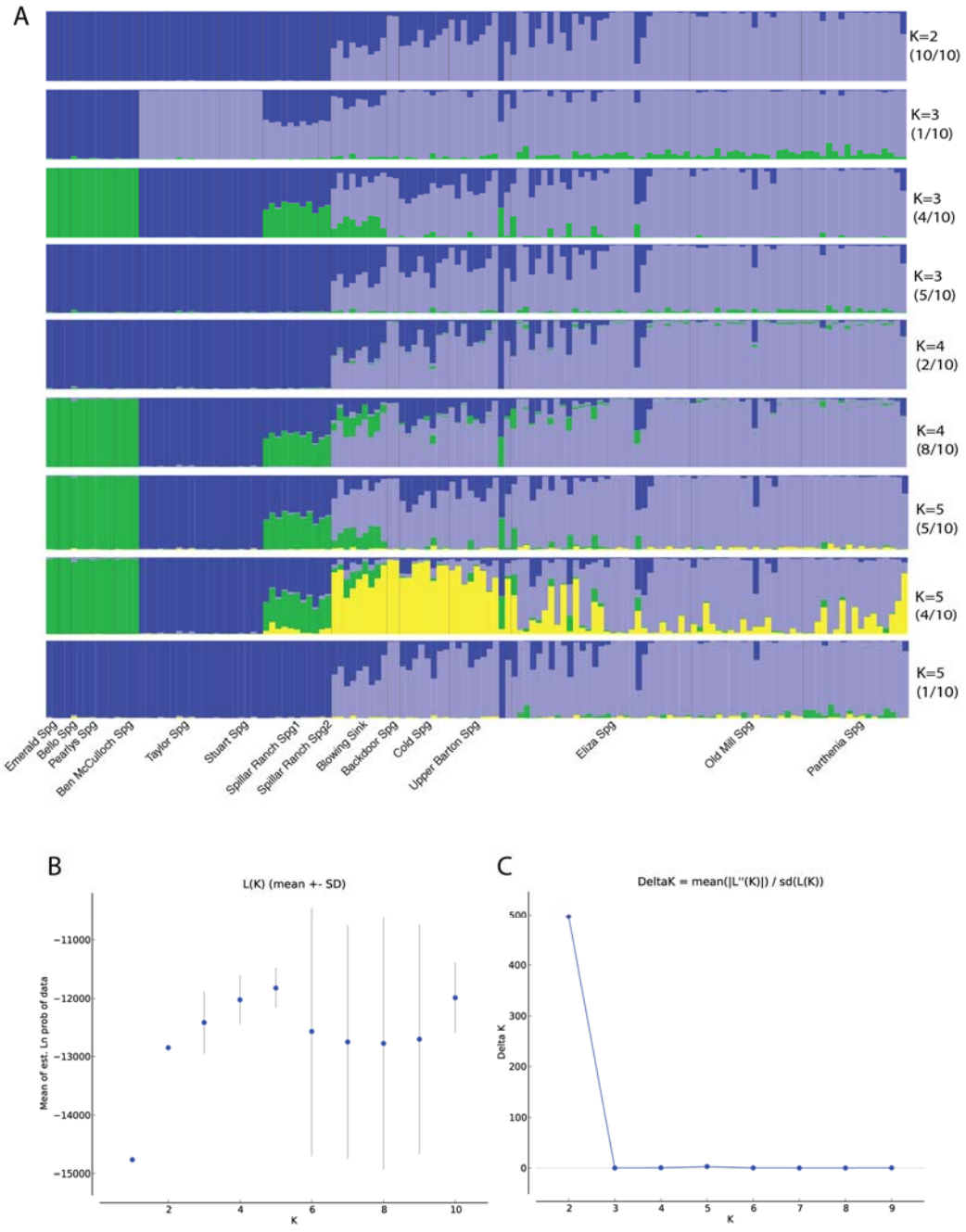

Figure 3. Summary plot of ancestry estimates from *Structure* analysis of 139 individual *E. sosorum* genotyped at 588 loci. Each individual in the dataset is represented by a single vertical bar that is colored by that individual's estimated membership proportion in each of *K* inferred clusters. The analysis consisted of 100,000 iterations following a burn-in of 10,000 iterations, assuming admixture and correlated allele frequencies among up to 5 populations. Ten runs were performed for each assumed value of *K*. A) Summary plot of *Q*, the estimated membership coefficient for each individual in each cluster, for *K*=2–5 (10 runs each). B) Plot of mean likelihood and variance per *K* value, showing a plateau around *K*=5.

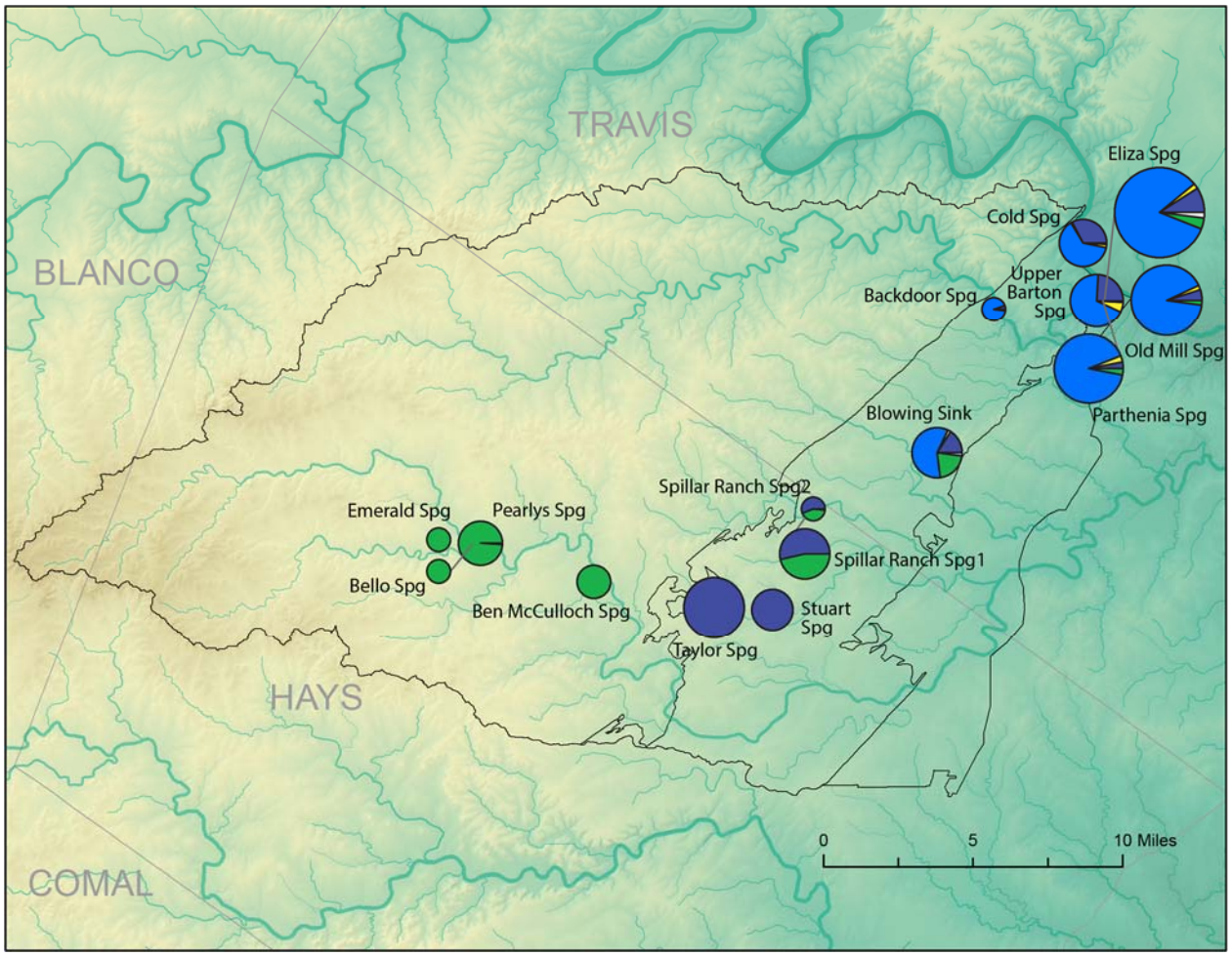

Figure 4. Results of Structure analysis of 139 individual *E. sosorum* genotyped at 588 loci showing the average individual membership coefficient (Q) for each sampling site for one run at K=5 (see bar plot, Fig. 3). The size of each pie diagram is proportional to the number of sampled individuals per site (N = 2–29).

FUTURE ANALYSES

Analyses that remain to be completed include basic descriptive statistics quantifying genetic variation as well as inferring ancestral population sizes and migration rates among subpopulations.

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